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BUTYRATE REGULATION OF DISTINCT MACROPHAGE SUBSETS: OPPOSING EFFECTS ON M1 AND M2 MACROPHAGES

Andrew D. Foey

School of Biomedical and Biological Sciences, University of Plymouth,
Drake Circus, Plymouth PL4 8AA, United Kingdom.

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ABSTRACT: ***Mucosal tolerance is central to efficient gastrointestinal tract function, tolerating food and commensal bacteria, whilst maintaining immune responsiveness to pathogens. Mucosal macrophages play a pivotal role in tolerance; whereas in inflammatory bowel disease, dysfunctional macrophages lead to tolerance breakdown, whereby commensals perpetuate inflammation. Macrophage subsets however, determine effector function: M1s are pro-inflammatory whereas M2s are anti-inflammatory/regulatory. In addition to commensal bacteria, butyrate, a short chain fatty acid probiotic metabolite, may also modulate macrophage-mediated tolerance. The human monocytic cell line, THP-1, was used to investigate butyrate immunoregulation in M1 and M2 macrophages, generated by monocyte differentiation in the presence of PMA or vitamin D_3, respectively. Butyrate modulation of LPS- and PGN-induced TNFα, IL-1β, IL-10 and NFkB was measured by sandwich ELISA and reporter gene assay, respectively. Data indicated butyrate suppresses LPS- and PGN-induced monocyte and M2 production of IL-1β and TNFα, M1-induced TNFα and IL-10 but failed to modulate M1-induced IL-1β. Additionally, butyrate augmented M2 IL-10 production, LPS- and PGN-stimulated M1 and LPS-induced M2 NFkB activity but failed to regulate PGN-induced M2 NF-kB. In conclusion, butyrate differentially regulates macrophage cytokine production and NFkB activation, which is subset-dependent and suggestive of a cautionary approach to butyrate use in treatment of mucosal inflammation.***

KEY WORDS: Butyrate, Cytokines, Inflammation, Macrophage

Corresponding Author: Dr. Andrew Foey, School of Biomedical and Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, United Kingdom; E-mail: andrew.foey@plymouth.ac.uk

INTRODUCTION

The immune system is capable of mounting localised inflammatory responses to gut pathogens through the recognition of conserved pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). The Toll-Like Receptors (TLRs) are capable of recognising broadly expressed bacterial PAMPs such as lipopolysaccharide (LPS) and peptidoglycan (PGN); commensal bacteria that are beneficial to the gut, however, may also express some of these PAMPs. Commensal bacteria are involved in gut mucosal development and function (Rhee et al., 2004). Inappropriate recognition of PAMPs expressed on commensals, by their cognate PRRs, however, could trigger a damaging inflammatory immune response. Thus, it is vital that recognition of bacterial PAMPs is tightly regulated that allows the host to tolerate commensal organisms yet maintain responsiveness to pathogens. The balance between luminal contents such as commensal and pathogenic bacteria, present in the intestinal microflora, and exposure of the mucosa to food-derived antigens is vital for the decision between mucosal tolerance (immune non-responsiveness) and immunity (Artis, 2008). Integral to this tolerance/activation decision and prevention of inappropriate immune responses to commensals, the gastrointestinal tract (GIT) has developed subtle modifications (Shenk and Mueller, 2007). This responsiveness to bacteria is regulated at many levels in cells of the gut mucosa, which include TLR expression, endogenous signal inhibitor expression, compartmentalisation of TLR expression and up-regulation of expression and activity upon dangerous insult.

Probiotic bacteria have long since been established to provide a beneficial effect on gut mucosal function. In addition to functions that facilitate epithelial cell growth, turnover and the production of mucus and anti-microbial peptides, probiotics and their metabolites have been described to exhibit an immuno-modulatory effect on the gut mucosa. One such group of probiotic metabolites includes the volatile short-

chain fatty acids (SCFA's) acetic, proprionic and butyric acids that predominate in the gastrointestinal tract (Campos et al., 2003). These SCFA's are produced by the fermentation of dietary fibre by anaerobic bacteria. The levels produced of each SCFA is dependent on location in the gut, bacterial population dynamics and diet; however, typical concentrations have been suggested to approximate 10mM acetate, 1.5mM butyrate and 2mM propionate (Scheppach et al., 1995; Cavaglieri et al., 2003). Butyrate has been described to play an important role in maintaining a healthy gut mucosa (Simpson et al., 2000), predominantly utilised by gut epithelial cells as a primary energy source, with a small amount passing to underlying tissue and into the bloodstream (Yin et al., 2001; Zapolska-Downar et al., 2004).

Butyrate exerts a variety of effects on gut epithelial cells and immune cells, essential for mucosal homeostasis thus allowing the host to tolerate food antigens and commensal microbiota whereas, at the same time, maintaining the capacity to initiate protective immune responses to pathogenic organisms. Butyrate plays an important role in mucosal barrier function; maintaining a healthy gut mucosa and modulating intestinal epithelial cell function by regulating differentiation, proliferation and enhancing tissue turnover, thus reducing the risk of inflammation and cancer (Brouns et al., 2002; Bocker et al., 2003). Additional observations have described butyrate to upregulate the anti-microbial peptide, LL-37 (Cathelicidin) expression in epithelial cells (Schwab et al., 2007) and expression of mucins (Willemsen et al., 2003). Impaired butyrate supply however, such as is the case in inflammatory bowel disease (IBD), where patients have an impaired ability to oxidise and utilise butyrate, underlies epithelial cell atrophy and an ensuing dysregulated immunity as a consequence of antigen overload in the sub-epithelial tissue (Roediger, 1980).

With respect to modulation of immune cell function, butyrate potently modulates both cells of the innate and adaptive immune system. It exerts an effect on T cell responses by suppressing antigen presentation through down-regulation of co-stimulatory molecules (B7-1), adhesion molecules (ICAM-1, LFA-3) (Bohmig et al., 1997), suppression of T cell alloresponses, resulting in prolonged allograft survival (Bohmig, et al., 1999) and facilitates a shift away from Th_1-dominant responses i.e. from pro-inflammatory to regulatory, anti-inflammatory responses (Cavaglieri et al., 2003). From current understanding of IBD, this would suggest a beneficial role for butyrate in the treatment of a Th_1/Th_{17}-driven pathology such as Crohn's disease (CD) but may be counter-intuitive with respects to the treatment of ulcerative colitis (UC), a Th_2-driven pathology. Butyrate effects differ with cell type, differentiation status, cytokine and inflammatory environment (Miller et al., 2005). In innate immunity, butyrate would appear to be an anti-inflammatory modulator, suppressing monocyte effector function by the inhibition of expression of pro-inflammatory cytokines such as TNFα?, IL1β and IL-12 (p40 and p70) with a corresponding up-regulation of the anti-inflammatory/regulatory cytokine, IL-10 (Saemann et al., 2000; Mahida et al., 2004). In contrast, however, upon co-culture of epithelial cells overlying monocytes; in the presence of an intact epithelial barrier, no cytokines could be induced; in the absence of this barrier, butyrate induced TNFa production and suppressed IL-10 (van Nuenen et al., 2005). Thus, it is suggestive that butyrate modulates both pro-inflammatory and anti-inflammatory/ regulatory effects. In addition, with respect to macrophages, butyrate has been suggested to alter macrophage differentiation, down-regulating differentiation markers and reducing phagocytic capacity (Millard et al., 2002). Butyrate both inhibited macrophage inflammatory mediators (TNFα, IL-6, CCL2, NO) and NFkB translocation (Soderberg et al., 2004); it was also found to down-regulate expression of TLR4, the LPS receptor, which indirectly impacts on NFkB activation and inflammatory cytokine production (Huuskonen et al., 2004).

The use of a butyrate enema in a rat model of induced colitis was observed to suppress inflammation and stimulate mucosal repair (Butzner et al., 1996), creating the rationale for this SCFA to be proposed for the treatment of IBD in humans (Bohmig et al., 1997; Venkatraman et al., 2003). With respect to the development of butyrate as an anti-inflammatory therapeutic, CD lamina propria mononuclear cell (MNCs) production of TNFa was suppressed as was pro-inflammatory cytokine production and nuclear translocation of the pro-inflammatory transcription factor, NFkB, induced by LPS activation of peripheral blood MNCs (Segain et al., 2000). Oral administration of butyrate to patients with active CD resulted in remission in 53% of patients with a corresponding suppression of IL-1β, NFkB and IL-12 (Di Sabatino et al., 2005). Surprisingly, butyrate administration to UC patients has produced encouraging results, suppressing mucosal inflammation by inhibiting NFkB activity in lamina propria macrophages (Breuer et al., 1997; Zapolska-Downar et al., 2004). Butyrate suppresses both activation of the inflammatory transcription factor, NFkB and binding efficiency to promoter binding sequences (Diakos et al., 2006; Huuskonen et al., 2004). In both monocyte-derived macrophages and gut lamina propria macrophages, this suppression of NFkB activity was shown to be as a consequence of stabilisation of the inhibitors, IkBα and IkBβ (Segain, 2000; Park et al., 2007; Zapolska-Downar et al., 2004; Yin et al., 2001). Thus, NFkB plays a central role in regulating the expression of a range of cytokines involved in gut inflammation (Neurath et al., 1998). Indeed, NFkB has been shown to be activated at inflammatory sites of patients with IBD (Segain et al., 2000). It is widely accepted that the anti-inflammatory effects of butyrate, that down-regulate pro-inflammatory mediators, are mediated by the suppression of IBD mucosal macrophages (Venkatranan et al., 2003).

Gut mucosal macrophages are central to deciding the fate of immune responsiveness to luminal antigens and bacteria. The nature of the response, i.e. activation or tolerance is

determined by the cells and molecules present which are themselves initiated by their local environment. Whereas most macrophages express a wide range of PRRs, intestinal macrophages are both functionally and phenotypically different from blood-derived monocytes. In the healthy gut, which is tolerant to luminal contents, intestinal lamina propria macrophages express MHC II and display a regulatory/anti-inflammatory phenotype characterised by phagocytic function, scavenger receptor expression (CD13, CD36), anti-inflammatory/regulatory cytokine/cytokine receptor expression (TGFβ, IL-10, TGFβR I and II), reduced responsiveness to PAMPs (low or absent expression of CD14 and TLRs) and fail to express integrin receptors, chemokine receptors, CD25, TREM-1, CD40, CD80, CD86 and CD89; this phenotype resembles that of the M2 macrophage subset (Smith et al., 2001; Smythies, 2005; Platt and Mowat, 2008) which are important in immune regulation and resolution of inflammation, during remission of IBD, by secreting anti-inflammatory cytokines (Mahida, 2000). Inappropriate presentation of luminal contents such as is observed in pathogenic insult or dysfunctional mucosal barrier results in a breakdown in tolerance and localised immune activation/inflammation. Breakdown in tolerance to commensal bacteria is suggested to contribute to the pathogenesis of Crohn's disease. In such inflammatory pathology, the lamina propria macrophages are characterised as $CD14^{hi}$, $TREM\text{-}1^{+}$, display an increased TLR responsiveness and expression of the pro-inflammatory cytokines IL-1β, IL-8, IL-12 and TNFα (Zareie et al., 2001). This phenotype resembles that of the M1 macrophage subset. Thus, the functional phenotype of macrophages present in the lamina propria is central in governing immune fate as tolerising or immune activatory/pro-inflammatory.

Butyrate exerts a suppressive effect on IL-12 which will subsequently decrease Th_1 production of the macrophage-activating cytokine, IFNγ, and consequently, pro-inflammatory cytokine production (Beck and Wallace, 1997). In line with this, butyrate also suppresses inflammation by inhibiting IFNγ signalling. IFNγ is upregulated in the mucosa of IBD patients (Stallmach et al., 1998) and is well established to be integral to activation and effector function of pro-inflammatory M1-like macrophages through the activation of STAT-1 (Klampfer et al., 2003). Butyrate may suppress STAT-1 activation through inhibiting the phosphorylation of its upstream activator, JAK2. Such a mechanism is thought to be important for butyrate exerting anti-inflammatory effects in IBD (Klampfer et al., 2003). Immunomodulation of IFNγ production and signalling, pro- and anti-inflammatory cytokines, TLR expression and monocyte differentiation by butyrate, thus directly affects mucosal macrophage functional phenotype. Differentiation status and effector function of gut mucosal macrophages is dependent on environmental factors encountered. As such, gut mucosal macrophages can be primed towards an anti-inflammatory/regulatory phenotype (M2) in normal healthy mucosa or towards a more pro-inflammatory phenotype (M1) during pathological insults upon barrier function being compromised. This dichotomy in macrophage effector phenotype partially explains the differential regulation of Th-driven responses; M1 cells effect either a Th_1 or a Th_{17}-driven response whereas M2 cells effect a Th_2-driven response (Mills et al., 2000).

With the development of butyrate administration to IBD sufferers, the immunopathological mechanisms driving CD and UC are contrasting. Taken into consideration that these responses may be driven by two divergent functional subsets of macrophage present in the gut mucosa, it is imperative to study the functional responses to butyrate treatment. Thus, the aim of this study was to investigate the effects of the short chain fatty acid, butyrate, on regulation of inflammatory responses induced in M1 and M2 macrophage subsets.

MATERIALS AND METHODS

Monocyte and macrophage culture

The human monocytic cell line, THP-1, was obtained from ECACC and routinely used for this study between passages 7 and 25. THP-1 cells were maintained in RPMI-1640 medium supplemented with 10%v/v foetal calf serum, 2mM L-glutamine and $100Uml^{-1}$ penicillin/100μg/ml streptomycin, here on referred to as R10 medium (Lonza, Wokingham, UK). The THP-1 NFkB reporter cell lines, THP-1Blue ($CD14^{lo}$) and THP-1Blue-CD14 ($CD14^{hi}$) were maintained in R10 medium in the presence of the selection antibiotics, zeocin ($200mgml^{-1}$) only ($CD14^{lo}$) or zeocin (200μg/ml) and blastocidin (10μg/ml) ($CD14^{hi}$)(Autogen Bioclear, Calne, UK). Cells were plated out at a density of $1x10^6$ cells/ml in R10 medium with $1x10^5$ cells per well in 96 flat-bottomed well tissue culture plates (monocyte cultures). Pro-inflammatory (M1-like) $CD14^{hi}$ macrophages and anti-inflammatory (M2-like) $CD14^{lo}$ macrophages were generated by differentiation of these monocytes in the presence of 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$-vitamin D_3 (Sigma-Aldrich, Poole, UK), for 7 days, respectively.

Activation of monocyte and macrophage cytokine production

Monocytes and macrophages were stimulated by the bacterial pathogen associated molecular patterns (PAMPs); 1μg/ml Peptidoglycan PGN (predominantly expressed in GM+ve bacteria and detected by TLR2/6 and NOD2) and 100ng/ml LPS (predominantly expressed in GM-negative bacteria and detected by TLR4) and cultured for 18 hours (determined as optimal time period for expression of all the cytokines TNFα, IL-1β and IL-10). After which, supernatants were harvested and stored at -20°C until required for assay by sandwich ELISA.

Regulatory effect of butyrate

Butyrate is thought to mediate its regulatory effects by suppression of the NFkB signalling pathway. To facilitate this

effect on intracellular signalling processes, sodium butyrate (But) (Sigma-Aldrich, Poole, UK) was added in culture to final concentrations of 0.1, 0.5, 1, 2 and 10mM as a pre-treatment for 4 hours prior to stimulation with the bacterial PAMPs, PGN and LPS. As a control experiment, to demonstrate a more physiologically relevant role for butyrate, cytotoxicity assays (MTT and trypan blue exclusion) were carried out on both monocytes and macrophages, up to 10mM butyrate. No significant reductions in viability were observed for the concentrations used in this study; viability was routinely >90%.

Cytokine measurement

The pro-inflammatory cytokines; TNFα and IL-1β and anti-inflammatory cytokine, IL-10, were analysed by sandwich ELISA using capture and detection antibodies commercially available from R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK. Protocols were followed as according to manufacturer's instructions and compared to standard curves, between the range of 7 to 5000pg/ml, using the recognised international standards available from NIBSC, Potter's Bar, UK. Colorimetric development was measured spectrophotometrically by an OPTIMax tuneable microplate reader at 450nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA).

NFkB activity measurement

NFkB activity was measured using a colorimetric reporter gene assay for secreted embryonic alkaline phosphatase, SEAP, associated with the stably-transfected reporter gene cell lines, THP-1Blue ($CD14^{lo}$) and THP-1Blue-CD14 ($CD14^{hi}$) (Autogen Bioclear, Calne, UK). Briefly, at the conclusion of the experimental incubation period, supernatant was harvested for analysis, fresh supernatant was incubated with Quantiblue colorimetric reagent (Autogen Bioclear, Calne, UK) at a dilution of 1:3 for 30 minutes at 37°C/5% CO_2. After which time, colorimetric development was measured spectrophotometrically by an OPTIMax tuneable microplate reader at 620nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA). The colour development being directly proportional to the reporter gene SEAP expression and hence NFkB activity.

Statistical analysis

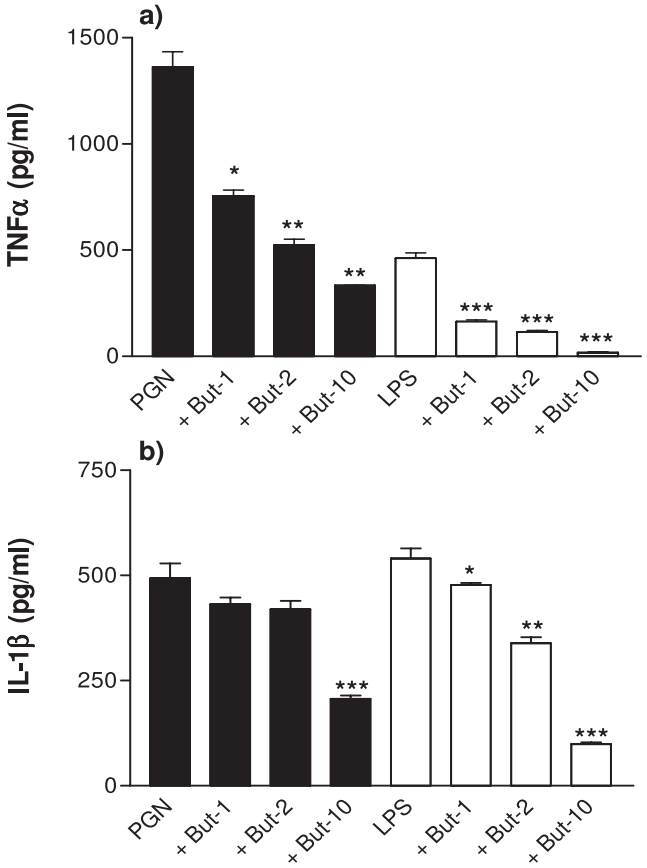
Measure of statistical significance was analysed by a paired Students' T test. Significance was set at $p<0.05$, where significant effects of butyrate compared to stimulus control were indicated as *$p<0.05$, **$p<0.01$ and ***$p<0.001$. NS denotes not significant.

RESULTS

Butyrate suppresses monocyte pro-inflammatory cytokines

Butyrate has been established to suppress peripheral blood-derived monocyte TNFα production. This experiment was undertaken to establish whether butyrate exerted the same effects on the monocytic cell line, THP-1, validating it as a useful model system to primary monocytes. Butyrate potently suppressed both PGN and LPS-induced TNFα; PGN response was suppressed from 1363±71pg/ml to 756±27pg (p=0.0119) and 335±2pg/ml (p=0.0032) for 1mM and 10mM butyrate respectively. LPS response was suppressed from 461±25pg/ml to 164±7pg/ml (p=0.0004) and 17±2pg/ml (p=0.0004) for 1mM and 10mM respectively (Fig 1a). Butyrate was less potent at suppression of IL-1β production; here, however, there was a differential sensitivity to suppression that was dependent on stimulus. PGN-induced IL-1β production displayed no significant suppression by butyrate until used at a concentration of 10mM (control levels of 493±35pg/ml suppressed to 207±8pg/ml, p=0.0004). LPS-induced IL-1β was more sensitive to suppression by butyrate with significant reductions observed for all concentrations used; from control levels of 540±24pg/ml to 477±5pg/ml (1mM, p=0.032), 339±14pg/ml (2mM, p=0.003) and 99±5pg/ml (10mM, p=0.0007).

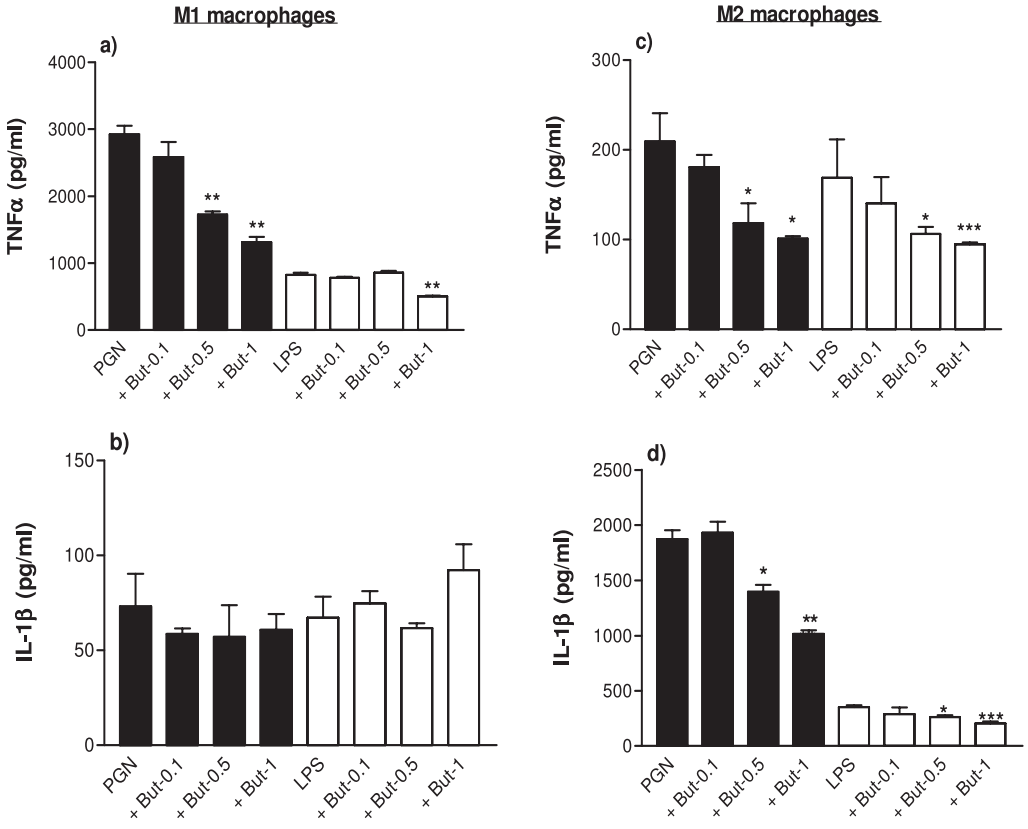
FIGURE 1. Butyrate suppresses monocyte pro-inflammatory cytokines. THP-1 monocytes were stimulated by 1μg/ml PGN (bold) or 100ng/ml LPS (unshaded) in the presence or absence of designated concentrations of sodium butyrate (mM). Cytokine production is expressed as the mean±SD in pg/ml for a) TNFα and b) IL-1β. Data displayed is representative of triplicate samples of n=3 replicate experiments.



Butyrate differentially regulates M1 and M2 macrophage pro-inflammatory cytokines

Butyrate has been suggested to have beneficial immunomodulatory effects in the gut mucosa for both homeostatic and inflammatory pathological conditions. To test this idea on macrophages, butyrate modulation of pro-inflammatory cytokines has been investigated for both M1-like (pro-inflammatory) and M2-like (anti-inflammatory) macrophages. Butyrate dose-dependently suppressed both M1 and M2 production of TNFα for both PGN and LPS stimulation. M1 PGN-induced TNFα was suppressed from control levels of 2923±130pg/ml to 1313±82pg/ml (p=0.0051) for 1mM butyrate (Fig 2a) and down to 746±8pg/ml at 2mM (p=0.0013, data point not shown). M1 LPS-induced TNFa was less sensitive to butyrate, only displaying suppression at 1mM (from control levels of 820±37 to 501±15 (p=0.0066) (Fig 2a); 2mM butyrate further reduced production to 364±13pg/ml (p=0.0029); data point not shown). M2 production of TNFa was sensitive to 1mM butyrate with PGN stimulus being suppressed from 209±32pg/ml to 101±3pg/ml (p=0.0484) and LPS suppressed from 169±42pg/ml to 95±2pg/ml, p=0.007 (Fig 2c). There was a differential response between M1 and M2 macrophages displayed for PGN and LPS-induced IL-1b production. M1, pro-inflammatory, macrophages displayed no significant modulation by 1mM butyrate for PGN (control 73±17pg/ml to 61±8pg/ml) or LPS (67±11pg/ml to 92±14pg/ml) stimulation (Fig 2b). M2 macrophage production of IL-1b, on the other hand, was sensitive to modulation by butyrate; PGN stimulus was suppressed from 1872±80pg/ml to 1014±36pg/ml (p=0.0025) and LPS stimulus from 352±18pg/ml to 205±19pg/ml (p<0.0001) by 1mM butyrate (Fig 2d). This data clearly shows that butyrate exerts differential effects on pro-inflammatory cytokine production by M1 and M2 macrophages, representative of mucosal inflammatory and mucosal anti-inflammatory/regulatory macrophages, respectively.

FIGURE 2. Butyrate differentially regulates M1 and M2 macrophage pro-inflammatory cytokines. M1 (a and b) and M2 (c and d) macrophages were generated by differentiating THP-1 monocytes with either 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. After which time, macrophages were stimulated by 1μg/ml PGN (bold) or 100ng/ml LPS (unshaded) in the presence or absence of the designated concentrations of sodium butyrate (mM). Cytokine production is expressed as the mean±SD in pg/ml for TNFα (a and c) and IL-1β (b and d). Data displayed is representative of triplicate samples of n=3 replicate experiments.

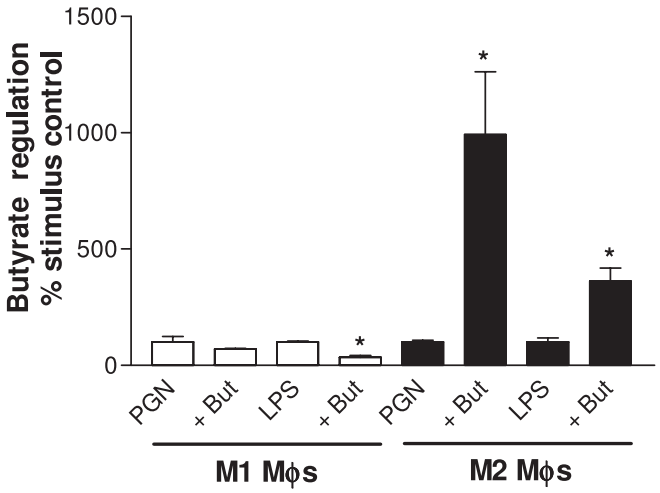


Butyrate differentially regulates anti-inflammatory cytokine, IL-10

Butyrate has been suggested to exert its anti-inflammatory effects via a range a mechanisms. One of these might include the augmentation of the anti-inflammatory cytokine, IL-10. The effect of butyrate on LPS- and PGN- stimulation on IL-10 production by M1 and M2 macrophages was examined. Butyrate, at a concentration of 1mM, exerted a differential effect on IL-10 production from M1 and M2 macrophages. Butyrate suppressed M1 production stimulated by both PGN (reduction in stimulation control levels to 70±3% control, p=0.1734) and LPS (reduction in stimulation to 34±9% control levels, p=0.013). Conversely, butyrate significantly augmented M2 macrophage IL-10 production. LPS-induced IL-10 was augmented to 361±57 % control level, p=0.0258 whereas PGN-induced IL-10 was augmented to 992±269% control stimulus levels, p=0.0293 (Fig 3). Thus, butyrate exerts both positive and negative effects on the production of

macrophage IL-10, dependent on macrophage subset present.

FIGURE 3. Butyrate differentially regulates anti-inflammatory cytokine, IL-10. M1 (unshaded) and M2 (bold) macrophages were stimulated with 1 μg/ml PGN or 100ng/ml LPS in the presence or absence of 1mM sodium butyrate (+But). Production of the anti-inflammatory cytokine, IL-10, is expressed as % production of stimulus control and is representative of triplicate samples of n=3 replicate experiments.



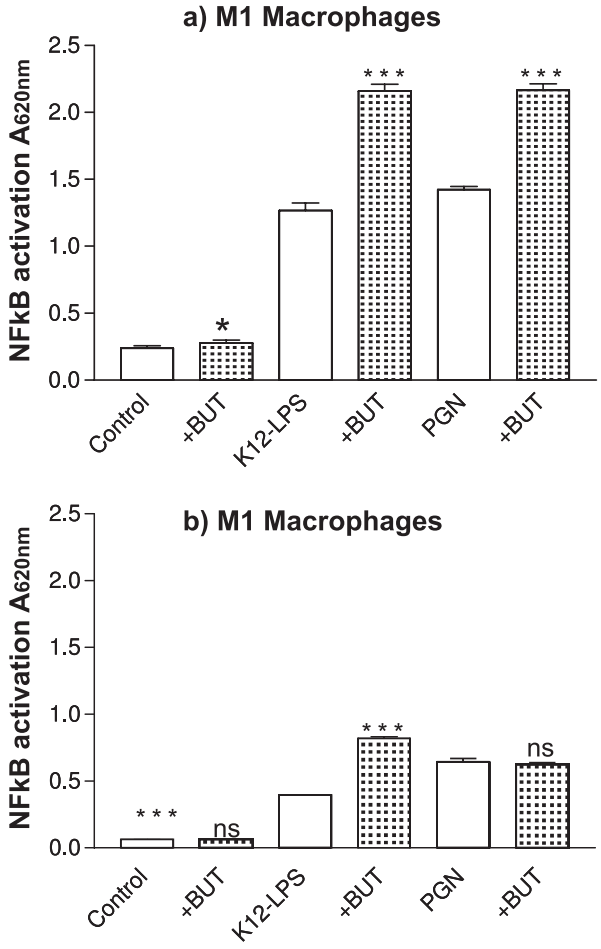
Butyrate fails to suppress PAMP-induced macrophage NFkB activation

Additionally, butyrate has also been suggested to exert an anti-inflammatory effect by inhibiting the transcriptional activity of the predominantly pro-inflammatory transcription factor, NFkB. The effects of butyrate on NFkB activity have been examined for M1 and M2 macrophages using a reporter gene construct-transfected cell line. In no situation did butyrate suppress NFkB activity in either M1 or M2 macrophages. Butyrate failed to modulate any NFkB activity in the absence of PAMP-stimulation. In M1 macrophages, butyrate augmented LPS and PGN activation of NFkB; butyrate augmented activity in $CD14^{hi}$ M1's by 70% ($p<0.0001$) and 52% ($p=0.0006$) for LPS and PGN respectively (Fig 4a). Butyrate augmented LPS-induced $CD14^{lo}$ M2 NFkB activity by 108% ($p=0.0003$) whereas PGN-induced activity was unchanged in $CD14^{lo}$ M2 macrophages ($p=0.243$, NS) (Fig 4b). Of note, was the fact that NFkB activation was highest in the proinflammatory $CD14^{hi}$ M1 macrophages and that this was unregulated upon treatment with butyrate.

DISCUSSION

Probiotics have been extensively studied for their potential to modulate immune function. They have been described to modulate some of these effects either directly through the physical interaction of these bacteria with their immune targets or indirectly through soluble secreted proteins or metabolites that result from fermentation of dietary

FIGURE 4. Butyrate regulation of PAMP-induced macrophage NFkB activation is subset- and stimulus-dependent. M1 $CD14^{hi}$ and M2 $CD14^{lo}$ macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1-NFkB reporter monocytes with either 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. M1 (a) and M2 (b) macrophages were stimulated with 1μg/ml PGN or 100ng/ml LPS in the presence (shaded) or absence (bold) of 1mM sodium butyrate. NFkB reporter activity is expressed as arbitrary absorbance units (A_{620nm}) and is representative of triplicate samples of n=3 replicate experiments.



components. The short chain fatty acid, butyrate, is produced from the metabolic breakdown of dietary fibre; this product also exhibits immunomodulatory capacity. This study demonstrates a differential immunomodulatory capacity for butyrate that is dependent on the macrophage subset being modulated. Thus butyrate can effect both a pro-inflammatory and an anti-inflammatory response determined by the predominant macrophage subset present in the gut mucosa.

Butyrate exhibited an anti-inflammatory immunomodulation of monocyte pro-inflammatory cytokines; dose-dependently suppressing PGN- and LPS-induced TNFα and IL-1β. This observation conformed to previous studies

(Saemann et al., 2000) but extended the findings in the context of suppression of these cytokines in response to predominantly Gram-negative bacteria (LPS stimulus) mediated through TLR4 and Gram positive bacteria (PGN stimulus), mediated through NOD2 and TLR2/TLR6.

With respect to butyrate modulation of macrophages, again other laboratories have described similar anti-inflammatory responses; this effect has been inferred to gut mucosal macrophages and suggested butyrate as an anti-inflammatory therapeutic treatment for IBD (Butzner, 1996; Segain, 2000; Di Sabatino, 2005; Park, 2007). Thus far, butyrate has exhibited limited success in the treatment of these diseases when used as an enema. This suggested that the macrophage influence was not as clear as formerly expected; something that was evident from the functional characterisation of macrophages as pro-inflammatory (M1) or regulatory/anti-inflammatory (M2), according to the activation stimuli or differentiation factors encountered (Mills et al., 2000; Foey et al., 2000; Anderson and Mosser, 2002; and reviewed in Mosser and Edwards, 2008). This study investigated the modulatory activity of butyrate on such macrophage subsets and found that the immunomodulatory capacity was determined by the differentiation of the monocyte into distinct subsets rather than stimulation through predominant Gram negative or Gram positive receptors, where butyrate exhibited similar effects on macrophages irrespective of stimulus being transduced through TLR4 or TLR2/6/NOD2. Butyrate suppressed LPS- and PGN-induced TNFa in both M1 and M2 macrophages whereas, IL-1β production was suppressed in M2 macrophages but failed to be suppressed in M1 macrophages. Pro-inflammatory M1 macrophages were not suppressed and remained inflammatory as a result of continued production of IL-1β, a cytokine highly prominent in the pathology of IBDs, which is counter-intuitive for developing this SCFA as a treatment for inflammatory bowel diseases. Additionally, butyrate has been suggested to induce/augment the production of anti-inflammatory cytokines such as IL-10. IL-10 suppresses both the production and bioactivity of pro-inflammatory cytokines such as IL-1β and TNFα (Fiorentino et al., 1991). This study both reinforced and contradicted these findings, that butyrate induces IL-10 (Saemann et al., 2000; Mahida et al., 2004). This anti-inflammatory effect was again, dependant on macrophage subset being studied. Butyrate suppressed pro-inflammatory M1 macrophage production of IL-10 in response to both LPS and PGN stimulation; albeit, these macrophages are very low producers of IL-10. On the other hand, LPS- and PGN-induced anti-inflammatory M2 macrophage production of IL-10 was significantly augmented. Thus, this early data was suggestive that the desired anti-inflammatory effect of butyrate was dependant on the macrophage subsets present in the inflammatory lesions to be treated; butyrate is highly beneficial when M2 subset predominates but may be less beneficial in the presence of the M1 subset; an inflammatory subset which predominates in CD. Of interest, TNFα and IL-1β have long-since been established to play a role in inducing IL-10 expression in monocyte/macrophages (Foey et al., 1998). Butyrate suppression of TNFα appeared to be independent of IL-10 expression/activity, as butyrate regulation of TNFα and IL-1β expression was observed at an earlier time point to that of IL-10 expression (data not shown). In addition, if IL-1β and TNFα directly induced IL-10 expression, then butyrate suppression of these pro-inflammatory cytokines would indirectly down-regulate IL-10. Thus, it is probable that butyrate regulates pro- and anti-inflammatory mediators separately via distinct pathways in these distinct macrophage subsets. Dissection of such discrete pro- and anti-inflammatory pathways in these subsets would prove useful for the informed development of anti-inflammatory regimens to be utilised in the treatment of IBD.

This differential sensitivity of M1 and M2 macrophage cytokine production to butyrate was suggestive of subtle differences between these two macrophage subsets with respect to reception, signalling and transportation of cytokines outside the cell. *In vitro*, our laboratory has observed that these subsets express similar TLR profiles with respect to detection of bacterial PAMPs (unpublished data). This was suggestive that there was either a difference in TLR profiles upon butyrate exposure, as butyrate has already been demonstrated to suppress TLR expression (Canto et al., 2006), or that M1 and M2 subsets exhibited differences in the signal transduction pathways that were utilised. It must be noted, however, that immunomodulation by butyrate is dependent on its availability after utilisation by epithelial cells, transport through the epithelial barrier and expression of butyrate receptors on the immune cells. SCFAs signal through a series of G-protein receptors (GPRs) where butyrate is a more selective ligand for GPR41 that is linked to IP_3-gated Ca^{2+} release, ERK-1/2 activation and the inhibition of cAMP accumulation by the coupled pertussis toxin sensitive Gi/Go protein (Brown et al., 2003). It is probable that these macrophage subsets express different profiles of these G-protein receptors, with profiles favouring anti-inflammatory function being predominant in the M2 macrophage subset.

With regards to signal transduction, butyrate is thought to modulate NFkB activity (Segain, 2000; Soderberg, 2004; Park, 2007). NFkB family is a group of transcription factors consisting of several proteins including NFkB1, NFkB2, p65 (rel A), c-rel and rel B (Tak and Firestein, 2001; Bonizzi and Karin, 2004). In its active conformation, NFkB exists as a heterodimer of these subunits, notably the p65/p50 dimer regulates inflammatory responses. The promoter regions of the proinflammatory cytokines, TNFα, IL-1β, IL-6 and IL-8 all express NFkB binding consensus sequences and have been shown to be highly regulated by NFkB; the anti-inflammatory cytokine, IL-10, does not possess NFkB binding consensus sequences and its expression is NFkB-independent (Bondeson et al., 1999). Inhibition of NFkB suppresses the expression of these pro-inflammatory cytokine genes. It was expected that butyrate would suppress these cytokines by inhibiting

NFkB activity. These data did not conform to this expectation; in fact, butyrate augmented both LPS- and PGN-induced NFkB activity in the pro-inflammatory M1 macrophages and LPS-induced activation in the anti-inflammatory/regulatory M2 macrophages. Interestingly, butyrate failed to augment PGN-induced activity in M2 cells. This lack of regulation of butyrate to the Gram-positive bacterial PAMP may be indicative of both the greater anti-inflammatory responsiveness of these macrophage cells and the regulatory nature of PGN expressed by gram-positive bacteria that include several probiotic strains.

Of note however, is the fact that the NFkB reporter gene assay used in this study did not discriminate between NFkB heterodimers or homodimers. The pro-inflammatory p65/p50 NFkB heterodimer may well have been inhibited and replaced by another NFkB dimer by either direct regulation or competition for binding to shared promoter sequences. In fact, the p50/p50 homodimer of NFkB has been demonstrated to exhibit anti-inflammatory or regulatory capability (Ziegler-Heitbrock, 2001; Saccani et al., 2006). Augmentation of LPS-induced NFkB activity observed in the anti-inflammatory subset may have been reflective of this differential NFkB subunit expression. This promoter activity may not discriminate between different forms of NFkB, hence the confusion in the real lack of discrimination of these NFkB data between M1 and M2 macrophages. Future investigations studying the effects of butyrate on NFkB will have to focus on subunit expression and utilisation in these distinct macrophage subsets and how they show differential sensitivity to regulation by the probiotic metabolite, butyrate. Finally, these NFkB data can also be suggestive that butyrate effects on macrophage cytokine expression are independent of NFkB activity. This does not necessarily conform to established studies however, describing butyrate suppression of NFkB but may be a valid conclusion when considering the differential pathways employed in specific effector functions of the macrophage subsets investigated in this study.

In conclusion, butyrate differentially regulates inflammatory cytokine expression in M1 and M2 macrophage subsets: suppressing M2 pro-inflammatory cytokines (TNFα and IL-1β) whilst augmenting the anti-inflammatory IL-10 and, conversely, suppressing M1 TNFα and IL-10 yet failing to modulate IL-1β. This failure to modulate M1 production of IL-1β is suggestive that suppression of TNFα is not enough with respect to control of inflammation and that pro-inflammatory effects can be driven by compensation for TNFa activities by IL-1β expression. Due to the overlapping activities and redundancy between these pro-inflammatory cytokines, it is likely that the development of successful clinical therapeutics will aim to suppress all pro-inflammatory mediators, preventing this compensation by other pro-inflammatory factors. Current use of butyrate in the treatment of IBD must be viewed with caution and may be only successfully used when both macrophage subset and cytokine profiling of the inflammatory pathology have been evaluated.

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